human PTEN.

C5 CONT.

28. The transgenic nematode of claim 25, wherein said mammalian PTEN is human PTEN.--

---

REMARKS

The present invention stems from Applicants' extremely important discovery that nematodes possess genetic pathways equivalent to those which control glucose metabolism in higher mammals. This discovery formed the basis for at least seven separate scientific publications by Applicants, including publications in the highly prestigious journals, *Science*, *Nature*, and *Genes & Development*. This discovery also facilitated the design of Applicants' assays for the identification of therapeutic compounds that target mammalian insulin signaling pathways, but which can be carried out in simple systems (such as nematode systems) that allow high throughput analysis.

The particular aspect of this invention claimed in the present application relates to methods for the identification of compounds capable of modulating the expression or activity of a *daf-18* or PTEN gene. Such compounds represent good candidates for therapeutics or therapeutic lead compounds useful for ameliorating or delaying impaired glucose tolerance conditions or obesity, or for increasing the longevity of a cell or organism. The presently claimed invention also relates to methods for diagnosing

impaired glucose tolerance conditions or obesity, as well as for determining the longevity of an organism based on the expression or activity of endogenous *daf-18* or PTEN genes.

Support for the Amendments

Applicants have amended claims 1-4, 8, 10-17, and 25 to focus on particular screens and screening systems. These amendments should not be construed in any way to indicate that Applicants agree with the current rejections in this case. Applicants reserve their right to pursue the canceled subject matter in future related applications.

Claims 1 and 2 have been amended to focus on screening methods carried out in nematode or mammalian cells. This amendment finds support, for example, in the specification at page 193, line 18 - page 195, line 8; page 196, line 19 - page 198, line 4; and page 198, lines 17-19 (for nematode cells); and page 190, line 20 - page 191, line 14 (for mammalian cells).

In addition, claims 3, 4, and 10-15 now specify that the claimed screens identify "candidate" compounds. This amendment is supported by the specification, for example, at page 197, line 13 (where the specification refers to "candidate" modulators of DAF-18/PTEN expression or activity).

The remaining claim amendments merely clarify claim language.

In addition, new claims 27 and 28 have been added. These new claims find support in the specification at page 26, line 20 and page 109, lines 10-16.

No new matter is added by these amendments.

Rejections under 35 U.S.C. § 112, first Paragraph

The only issue to be addressed in this case is a rejection of claims 1-5, 8-23, and 25-26, under 35 U.S.C. § 112, first paragraph, based on the assertion that Applicants' specification does not enable the presently claimed *daf-18* or PTEN screening methods, or the presently claimed transgenic nematodes. This rejection is based on the following three grounds: (1) that the specification fails to enable the isolation of *daf-18* or PTEN promoters; (2) that the specification fails to provide guidance for the construction of a transgenic nematode expressing a mammalian PTEN gene; and (3) that the specification fails to show that mammalian PTEN is associated with the onset of impaired glucose tolerance, obesity, or longevity in mammals. This rejection is respectfully traversed.

*The Nematode daf-18 and Human PTEN Promoters Are Enabled by the Present Specification*

The first basis for the § 112 rejection involves the assertion that the specification fails to describe the nematode *daf-18* and human PTEN promoters. This basis for the rejection is respectfully traversed. As of the filing date of the present specification, such promoters could be readily isolated using only standard techniques of molecular biology. For example, at the time the application was filed, genomic library screening

methodologies, PCR techniques, and genomic sequence database searches were commonly used by molecular biologists to obtain 5' flanking regions of a desired gene, once the coding region of that gene was available.

As evidence of the ease with which the *daf-18* and PTEN regulatory regions may be (and, in fact, were) isolated, Applicants have previously demonstrated that standard techniques were used to clone the *C. elegans daf-18* promoter. As attested to in the Declaration of Dr. Gary Ruvkun submitted July 10, 2000, the *daf-18* promoter was cloned using methods well known to a practitioner in the field of molecular biology. In addition, Applicants have also previously submitted evidence that the PTEN regulatory region was isolated and its sequence made available on the Entrez sequence database shortly after the cDNA for that human gene was cloned. In view of these facts, Applicants submit that there is no basis to believe that isolation of either the *daf-18* or PTEN promoters would, or did, involve undue experimentation. This basis for the enablement rejection should be withdrawn.

*The Specification Enables the Production of Transgenic Nematodes*

The second basis for the § 112 rejection is based on the assertion that the specification fails to enable a transgenic nematode expressing a nematode *daf-18* or human PTEN gene. In particular, the Office states that the specification fails to show that a DNA fragment encoding either *daf-18* or PTEN could be introduced into a nematode to

produce the required phenotype of such transgenic animals. This basis for the rejection is also respectfully traversed.

Applicants submit that the present specification provides a teaching that is more than adequate to enable the production of transgenic nematodes expressing either a *daf-18* or a PTEN gene. The specification, for example, at page 38, provides a reference (Mello *et al.* EMBO J. 10:3959-3970, 1991) for generating transgenic *C. elegans*; such methods were standard in the field of nematode genetics at the time the present application was filed. In addition, the sequences necessary for transgenic nematode production were provided by Applicants' specification. The *daf-18* gene sequence was provided in the present specification (see, for example, Figures 40A and 40B), and the PTEN gene sequence, as noted in Applicants' specification at page 109, was publicly available (for example, in the Entrez sequence database at Accession Number U93051). Using this teaching, one of skill in the art could readily generate a transgenic PTEN nematode.

As further evidence of the ease with which transgenic nematodes could be produced given Applicants' disclosure, Applicants submit herewith a Declaration from Dr. Gary Ruvkun. There, Dr. Ruvkun describes experiments demonstrating that, using *daf-18* and PTEN genes in combination with the injection techniques described in the present specification, transgenic *daf-18* and PTEN nematodes were successfully generated. In particular, as outlined in this Declaration, Dr. Ruvkun's laboratory constructed *daf-18* and PTEN "minigenes" using cDNAs and native *daf-18* 5' flanking

sequence (approximately 1.0 kb) and 3' flanking sequence (approximately 2.4 kb). These minigenes were constructed by standard PCR overlap extension techniques using nested primers, and restriction enzyme digests confirmed the identity of the final PCR products.

Standard techniques referred to in Applicants' specification at page 38 were then used to generate the transgenic nematodes (Mello *et al.*, EMBO J. 10:3959-70, 1991). Specifically, the minigenes were co-injected with a plasmid encoding green fluorescent protein (GFP) under the control of the *sur-5* promoter (*sur-5*:GFP) into *daf-2*; *daf-18 C. elegans*. *sur-5*:GFP is a widely expressed GFP that serves as a convenient co-injection marker for identification of transgenic *C. elegans*. Double mutant *C. elegans* were chosen to be injected so that rescue of the *Daf-d* phenotype (i.e., no dauer formation at 25°C) of the mutants could be easily assayed. Transgene rescue of *daf-18* would result in a phenotypic reversion of the injected *daf-2*; *daf-18* strain to that of a *daf-2* phenotype, resulting in a high percentage of GFP-expressing *C. elegans* that formed dauers at 25°C, but not at lower temperatures, for example, 20°C. *sur-5*:GFP was injected alone as a negative control, and a *daf-18* rescuing genomic PCR fragment was coinjected with *sur-5*:GFP as a positive control.

GFP-expressing F1 *C. elegans* were picked for egg lay at 25°C, and F2 *C. elegans* were then scored for GFP expression and the dauer phenotype. Results of the injections are shown below in Table 1.

**Table 1. Human PTEN rescues *daf-18(mg198)***

| Injected Transgene | # GFP-positive *C. elegans* | # dauers | % rescue |
|---|---|---|---|
| none | 53 | 0 | 0 |
| *daf-18* genomic | 16 | 16 | 100 |
| *daf-18* minigene | 17 | 17 | 100 |
| PTEN minigene | 33 | 33 | 100 |

Percent rescue was calculated by dividing the number of dauers by the number of GFP-positive *C. elegans*.

As attested to by Dr. Ruvkun in the accompanying Declaration, the results shown in Table 1 indicate that both the *daf-18* transgene and the PTEN transgene mediate the rescue of a *daf-18* mutant at a level of 100%. The negative control in these experiments resulted in 0% rescue of the *daf-18* mutant, while the positive *daf-18* genomic control also yielded 100% rescue.

In view of these results, there can be no question that the construction of transgenic nematodes is enabled by the teaching of Applicants' specification. In addition, in response to the Examiner's concern regarding functional similarity, there can also be no question that DAF-18 and PTEN have similar, if not identical, activities.

Finally, on this issue, Applicants note the assertion made in the Advisory Action that the use of a mutant recipient nematode for the production of a transgenic animal somehow calls into question the enabling nature of the specification. Applicants

respectfully disagree. As demonstrated by Applicants, *daf-18* and PTEN transgenic nematodes can be successfully generated, and there is no scientific basis of which Applicants are aware that would suggest that an analogous approach using a wild-type recipient nematode would be any less effective. This second basis for the enablement rejection should be withdrawn.

*PTEN Is Involved in Impaired Glucose Tolerance, Obesity, and Longevity in Mammals*

The third and final basis for the § 112 rejection focuses on the statement in the Office Action that presently claimed screens lack enablement because no evidence exists that the mammalian *daf-18* homolog, PTEN, is associated with the onset of impaired glucose tolerance, obesity, or longevity in mammals. This basis for the rejection may also be withdrawn.

On this issue, the Examiner is first directed to a previously submitted reference by Maehama et al. (J. Biol. Chem. 273:13375-13378, 1998). As pointed out by Applicants, this reference first indicated the involvement of PTEN in insulin signaling in human cells.

Confirming and extending these results are the later publications submitted herewith as Exhibits I and II. The first reference is by Nakashima et al. (J. Biol. Chem. 275: 12889, 2000; Exhibit I) and shows that excess PTEN activity leads to inappropriate insulin signaling in mammalian cells, just as indicated by Applicants in the present

specification. In the Nakashima experiments, overexpression of PTEN in differentiated mammalian adipocytes inhibits insulin-induced 2-deoxy-glucose uptake, translocation of the GLUT4 transporter to the plasma membrane, and cellular membrane ruffling, all events normally associated with insulin stimulation. In addition, Nakashima demonstrates that microinjection of those same mammalian adipocytes with an anti-PTEN antibody reverses the PTEN effects tested; specifically, upon injection of the anti-PTEN antibody, basal and insulin-stimulated GLUT4 translocation were increased. These results again demonstrate the role of PTEN in mammalian insulin signaling.

In addition, Exhibit II is a publication by Iida et al. (Anticancer Research 20:1901-1904, 2000). In this publication, Iida demonstrates that a human patient carrying a heterozygous PTEN gene mutation that results in decreased levels of PTEN is hypersensitive to insulin. This was shown by administering a bolus of glucose to the patient and measuring plasma glucose levels. Compared to normal control subjects, the patient with decreased PTEN activity exhibited a more rapid clearance of blood glucose, indicating that the patient was insulin hypersensitive.

This same patient's insulin hypersensitivity was further demonstrated by a euglycemic hyperinsulinemic clamp study. Briefly, insulin was injected into the patient to raise insulin serum levels to those necessary to maintain euglycemia. Exogenous glucose was also injected into the patient to achieve a desired steady state serum insulin concentration and to maintain blood glucose levels within the euglycemic range. During

maintenance of euglycemia, the steady state glucose infusion rate was higher for this patient than the rates needed for healthy subjects, again indicating insulin hypersensitivity in the patient with decreased PTEN levels.

In sum, the above reports demonstrate the role of PTEN in mammalian insulin signaling and therefore its role in impaired glucose tolerance conditions in mammals.

With respect to insulin conditions involving obesity, Applicants submit that a number of lines of evidence indicate that the *daf* and PTEN genes play roles in obesity conditions and that the presently claimed screening methods could indeed be exploited for the identification of anti-obesity compounds.

On this issue, Applicants again direct the Examiner's attention to the Declaration of Dr. Gary Ruvkun, filed October 26, 1999. There, Dr. Ruvkun points out that dauer arrest of various insulin signaling pathway mutants can be rescued by expression of a protein which complements the mutation, and that such a rescue also results in the loss of fat in the rescued animals. For example, *C. elegans daf-2* mutants are normally dauer-arrested and exhibit increased fat accumulation when compared to their wild-type counterparts. When the *daf-2* mutants are rescued from dauer arrest by mutation of the *daf-18* gene, and therefore from an impaired glucose tolerance condition, they also exhibit lower fat accumulation levels. Similar results are observed in *C. elegans age-1* mutants. These mutants also exhibit increased fat accumulation as dauers, and, upon rescue from dauer arrest by expression of the wild-type AGE-1 protein, similarly exhibit lower fat

accumulation levels.

With respect specifically to the role of *daf-18* and PTEN in obesity, Applicants refer the Examiner to Figs. 38A-38F of Applicants' specification, where it is demonstrated that the *C. elegans* homolog of PTEN, *daf-18*, modulates the level of fat accumulation in *C. elegans*. The data presented in this figure show that *daf-18* suppresses the fat accumulation phenotype of an *age-1* null mutant, providing direct evidence that *daf-18* is involved in the regulation of fat levels in nematodes. Applicants submit that, due to the structural and functional similarity between DAF-18 and PTEN (as discussed above) and the known role of PTEN in the regulation of insulin signaling in mammalian cells, PTEN, like *daf-18*, would be fully expected to regulate fat accumulation in mammals and could successfully be used in the presently claimed screens for obesity-related compounds.

Moreover, Applicants submit that the relationship between impaired glucose tolerance conditions and fat accumulation in mammals is well established in the medical literature. The inability of a mammal to regulate metabolism results in a shift of its metabolism away from burning energy and toward metabolism of fat. The metabolism of fat, in turn, leads to other conditions in mammals, including obesity.

Finally, to address the contention in the Office Action that the specification fails to show that PTEN regulates longevity in mammals, Applicants direct the Examiner to the evidence presented in the present specification demonstrating that *daf* genes regulate

longevity in nematodes. In particular, Applicants refer the Examiner to pages 103-107 of the specification, where evidence of the role of *daf* genes in longevity is described. There, Applicants indicate that weak *daf-2* and *age-1* mutants that do not arrest at the dauer stage nevertheless live much longer than their wild-type counterparts. In addition, a *daf-18* mutation supresses the long life span of the *daf-2* and *age-1* mutants. These results demonstrate that the insulin signaling pathway, of which *daf-18* is a member, can modulate longevity. The specification further states that *age-1* null mutants are characterized by their longevity phenotype. These *age-1* null mutations are suppressed by the mutant, *daf-18(e1375)*. Again, these results clearly demonstrate that *daf* genes, including *daf-18,* play a role in the regulation of longevity in nematodes.

With respect to the role of PTEN in longevity, Applicants direct the Examiner to Table I above and submit that, due to the functional similarity between DAF-18 and PTEN in the regulation of dauer arrest and longevity in nematodes, one skilled in the art would predict that PTEN also functions to regulate longevity in mammals. Accordingly, both *daf-18* and PTEN represent useful genes for identifying candidate therapeutic compounds for increasing longevity, as presently claimed.

In sum, Applicants have demonstrated that *daf-18* and PTEN are functionally interchangeable and have established that these genes function to regulate impaired glucose tolerance conditions, fat accumulation, and longevity, providing strong evidence that screens involving either of these genes would successfully identify candidate

compounds involved in such conditions. Applicants submit that this final basis for the § 112 rejection may be withdrawn.

## CONCLUSION

Applicants submit that the claims are in condition for allowance, and such action is respectfully requested.

If there are any charges, or any credits, please apply them to Deposit Account No. 03-2095.

Respectfully submitted,

Date: 31 January 2001

Karen L. Elbing, Ph.D.
Reg. No. 35,238

Clark & Elbing LLP
176 Federal Street
Boston, MA 02110
Telephone: 617-428-0200
Facsimile: 617-428-7045

21559
PATENT TRADEMARK OFFICE

\\Ntserver\documents\00786\351xxx\00786.351004 Preliminary amendment.wpd